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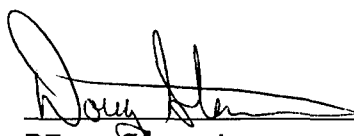
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## **Introduction:**

Epidemiological evidence suggests breast cancer risk is closely related to development of the mammary gland. Additionally, development of the mammary gland through fetal life, puberty, pregnancy and post-lactational regression involves cellular processes such as differentiation, proliferation and apoptosis(1-3). Protein kinases are key regulatory molecules of these cellular processes(4). Several protein kinases have been shown to contribute to mammary gland development and breast cancer, including c-erb-B2/Neu, the epidermal growth factor (EGF) receptor, c-Src and Met(4-11).

To identify other kinases involved in both of these processes, we performed RT-PCR using degenerate oligonucleotides to catalytic subdomains VII and IX conserved in all protein kinases. Forty-one kinases were identified after screening several transformed mammary epithelial cell lines and developmental time points in the mammary gland by this method. Three of these kinases were novel, one of which, H51, does not fit into any known kinase subfamily and has closest homology to a serine/threonine kinase in yeast with unknown function.

The H51 PCR product obtained from this screen was used to isolate a full-length cDNA. The kinase encoded by this cDNA was renamed from H51 to Krct and will henceforth be referred to as such. After isolation of full length *Krct*, analysis of its expression pattern, chromosomal localization and verification of its phosphotransferase activity were performed and published(12). Since many kinases are involved in cellular proliferation and differentiation, we hypothesize that Krct may also be involved in these processes. To investigate this hypothesis, *in vitro* and *in vivo* proliferation and differentiation assays will be performed.

## **Specific Aims:**

### **Aim 1: Determine the role played by Krct in proliferation.**

- A. Generate antisera specific for Krct.**
- B. Characterize the expression of Krct *in vitro* as a function of cell proliferation.**
- C. Overexpress Krct to assess its effects on proliferation.**
  - i. Assessing the effects of Krct overexpression on proliferation *in vitro*.**
  - ii. Assessing the effects of Krct overexpression on proliferation *in vivo*.**

### **Aim 2: Determine the role played by Krct in differentiation.**

- A. Characterize the expression of Krct in an *in vitro* differentiation system.**
- B. Overexpress the novel kinase, Krct, to assess its effects on differentiation *in vitro*.**
- C. Overexpress the novel kinase, Krct, to assess its effects on differentiation *in vivo*.**

### **Aim 3: Determine the potential role played by Krct in carcinogenesis in the mammary gland.**

- A. Assessing the effects of Krct on transformation *in vitro*.**
- B. Assessing the tumorigenicity of Krct overexpressing cells.**
- C. Determine the effects of Krct overexpression on carcinogenesis *in vivo*.**

### **Task1. Determine the role played by Krct in proliferation: months 1-36.**

- Generate antisera to Krct: months 1-14.
- Generate pTetO.Krct and pTetO.LacZ constructs: months 1-7.
- Characterize Krct expression during proliferation in HC11 cell: months 8-16.
- Generate and characterize stable transfectants in HC11 cells: months 17-24.
- Perform Northern and Western analysis to assess proliferation rates: months 25-36.
- Perform FACS and 3H-Thymidine studies to assess proliferation rates: months 25-36.
- Create MMTV.Krct transgenic construct: months 1-6.
- Create founder mice for transgenic construct: months 7-10.
- Characterize changes in proliferation rates in transgenic mice by *in situ* hybridization, whole mount analysis, and hematoxylin and eosin stained histologic sections: months 10-36.
- Characterize changes in proliferation rates in transgenic mice by Northern and Western analysis: months 12-36.

**Generate antisera to Krct (months 1-14).** Since Krct regulation is likely to occur at the translational level due to upstream AUG codons in the 5'-untranslated region (UTR) in its mRNA, it will therefore be important to determine Krct protein levels. Therefore, antisera were generated to two different regions of the protein. GST-Krct fusion constructs were generated to the middle and C-terminal regions of Krct. These constructs were expressed in *E. coli* and purified using glutathione sepharose beads and cleaved with a peptidase supplied by the manufacturer. The liberated and purified Krct peptides were injected into two rabbits each. The resulting antisera were then affinity purified and tested for its ability to recognize Krct protein by Western analysis and its ability to immunoprecipitate (IP) Krct. All four antisera generated were able to detect Krct by Western analysis and IP Krct (Fig. 1). With these antisera, we will be able to determine if protein levels are cell cycle regulated similar to Krct mRNA.

**Characterize Krct expression during proliferation in HC11 cell (months 8-16).** To determine Krct's role in proliferation, Krct mRNA expression levels in actively growing, quiescent, and synchronized cells have been determined. Krct is expressed at similar levels in actively growing cells and confluent cells in two different mammary epithelial cell lines, HBI2 and 16MB9A cells. However, upon serum starvation in these cell lines, Krct mRNA levels significantly decrease. After refeeding, Krct levels remain low until just after Cyclin A induction where Krct expression dramatically increases (Fig. 2). Despite the observation that Krct mRNA levels don't change when cells become quiescent, its change in expression upon serum starvation/release demonstrates Krct is likely a cell cycle regulated gene. Additional Northern analysis needs to be performed to follow up this observation. Since Krct expression levels do not peak during this time course, serum starve/refeed experiments need to be performed again and cells harvested at later times.

Protein expression levels of Krct during a serum starve/refeed experiment of HC-11 cells were determined. Krct levels did not change during the time course. This is not consistent with mRNA levels seen in HBI2 and 16MB9A cells. This brings into question whether Krct is regulated during the cell cycle. Further synchronization experiments analyzing Krct protein and mRNA from matched samples in these three cell lines will need to be performed to address these disparate results.

**Generate pTetO.Krct construct (months 1-7).** Since most genes regulated by the cell cycle are themselves regulators of the cell cycle, over-expression studies will be performed to

determine if Krct over-expression perturbs the cell cycle and therefore alters proliferation rates. To inducibly over-express Krct, the tetracycline-inducible plasmid-based expression system will be used. A modified Krct cDNA was generated for over-expression. PCR-based truncation of the 5'UTR was performed to remove all upstream AUG codons and place an in-frame stop codon upstream of the initiation codon. This modified cDNA was subcloned into a plasmid between the TetO regulatory sequence and an SV40 3'UTR containing an intron for more efficient mRNA expression in mammalian cells. This expression construct will be used to generate inducible, stable clones of Krct for proliferation and differentiation assays.

**Create MMTV.Krct transgenic construct (months 1-6) and create founder mice for transgenic construct (months 7-10).** To further study Krct's potential role in proliferation, transgenic animals are to be generated with full length Krct cDNA placed under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter. The MMTV-Krct transgenic construct has been created and injected into oocytes from FVB mice. Injections were performed by the University of Pennsylvania Transgenic Mouse Core Facility. Southern analysis of mice generated confirmed the generation of four transgene positive animals, two females and two males. One female animal died in the process of breeding these transgene positive animals with wild type FVB mice to verify germline transmission of the transgene in these four potential founders. Autopsy analysis of this female shows her to have had only one kidney and to have been pregnant at the time of death. Presumably the stress from the pregnancy on her only kidney resulted in her death and not an effect from the transgene. The other three transgene positive animals are able to pass the transgene.

The next step in analysis is to determine the expression levels of mice derived from each of these lines. This will be done by Northern analysis and if necessary, by a RNase A protection assay. A probe will be used which overlaps the junction of the Ras leader sequence and Krct cDNA. This will identify both endogenous and exogenous expression levels simultaneously by Northern and RNase protection analyses.

**Generate and characterize stable transfectants in HC11 cells: (months 17-24).** Generation of inducible Krct clones has proven to be difficult. The pTetO.Krct construct was transfected into HC-11 cells previously transfected with pTetO.rtTA which has previously been demonstrated to inducibly express rtTA in a doxycycline –dependent manner. After puromycin selection for pTetO.Krct transfectants, clones were isolated, expanded and characterized for the ability to overexpress Krct. Cells were induced with 1 ug/ml of doxycycline for 48 hours. After screening over 30 Krct clones by Western analysis, we have identified one clone that overexpresses Krct by approximately 5 fold (Fig. 3). One clone is not sufficient to prove any potential phenotype observed is due to transgene expression and not a result of disrupting the expression of a gene at the integration site, therefore at least one more clone will need to be generated to verify observations made with the first clone.

## **Task2: Determine the role played by Krct in differentiation: months 1-36.**

- Characterize Krct during HC11 cell differentiation: months 8-17.
- Perform Northern and Western analysis to detect changes in H51 and B-casein levels: months 18-26.
- Assess Krct overexpression on differentiation *in vivo*: months 10-36.
- Characterize changes in differentiation in transgenic mice by Northern and Western analysis: months 10-36.

- Characterize changes in differentiation in transgenic mice by *in situ* hybridization, whole mount analysis, and hematoxylin and eosin stained histologic sections: months 10-36.

**Characterize Krct during HC11 cell differentiation. Perform Northern and Western analysis to detect changes in H51 and B-casein levels: (months 8-26).** Over-expression of protein kinases has been shown to affect differentiation of the mammary gland. To investigate the role of Krct in differentiation, we will analyze Krct expression during HC-11 differentiation, a mammary epithelial cell line which will express  $\beta$ -casein when stimulated with prolactin, dexamethasone, and insulin. Protein extracts and RNA samples have been made in the lab. Cells were stimulated with 1ug/ml of both prolactin and dexamethasone and 10% fetal calf serum. Cells were harvested for RNA and protein at 0 hours while actively growing, and 6hrs, 1,2,3,4,5,7, and 10 days post induction. Cells were all harvested during the same passage. Krct expression was been determined by Western analysis. Krct protein levels appear to increase slightly through day 4 of induction and decrease thereafter to levels significantly lower than that in uninduced states. Northern analysis of Krct and  $\beta$ -casein expression during differentiation will be performed to determine the differentiation states of the cells at each time point.

Once the expression profile of Krct relative to  $\beta$ -casein induction is determined during the normal differentiation of HC-11 cells, the affects of Krct over-expression on HC-11 differentiation will be examined using the same inducible system described in Task 1. Additionally, affects of over-expression of Krct *in vivo* will be determined using the same MMTV-Krct transgenic animals created for Task1.

**Task3: Determine the potential role played by a novel serine/threonine kinase in carcinogenesis in the mammary gland: months 1-36.**

- Generate and characterize 3T3 fibroblast cell lines containing pTetO.Krct and pTetO.LacZ constructs: months 7-14.
- Perform focus formation study: months 15-22.
- Perform soft agar experiment: months 15-22.
- Determine the effects of Krct overexpression on carcinogenesis by injecting nude mice: month 12
- Characterize changes in tumor status of nude mice by gross dissection, and hematoxylin and eosin stained histologic sections: months 10-36.
- Characterize changes in tumor status of transgenic mice by gross dissection, and hematoxylin and eosin stained histologic sections: months 7-36.

**Generate and characterize 3T3 fibroblast cell lines containing pTetO.Krct and pTetO.LacZ constructs: months 7-14.** Several kinases have been demonstrated to function as oncogenes in human cancers and murine animal models of mammary gland carcinogenesis(11, 13-16). To investigate if Krct has a role in mammary gland carcinogenesis, we will use the inducible constructs to be generated in Task 1. These inducible cells will be tested for their ability to form foci, grow in soft agar and form tumors in nude mice. Since reagents to be used for this task were previously described in Task 1 and no further work in this task has been completed, there is nothing to report for Task 3 at this date. The generation of Krct stable clones will be performed in the near future according to schedule.

**Perform focus formation study: (months 15-22).** During the time it has taken to generate one inducible Krct clone, a focus formation assay was performed by transiently transfecting a CMV-Krct construct into 3T3-L1 cells. Cells were cultured until confluent and fed once every two days for two weeks and stained. No foci were observed on plates transfected with CMV-Krct or with the empty vector as a control. This experiment will need to be repeated since no foci formed. It is possible that 3T3-L1 cells may not transform as easily as NIH3T3 cells. Additionally, overexpression of Krct may not be able to transform 3T3 cells alone. Therefore, the addition of a known oncogene may be necessary. In which case Krct will be assessed for its ability to modulate the transformation frequency of the known oncogene used.

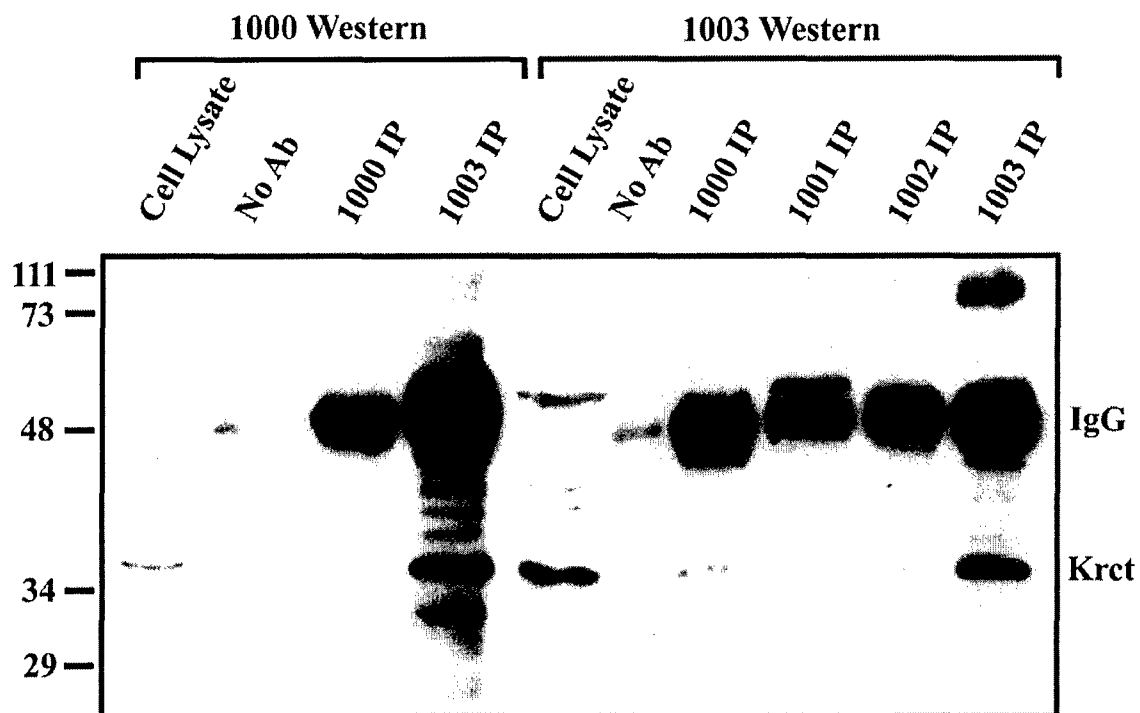
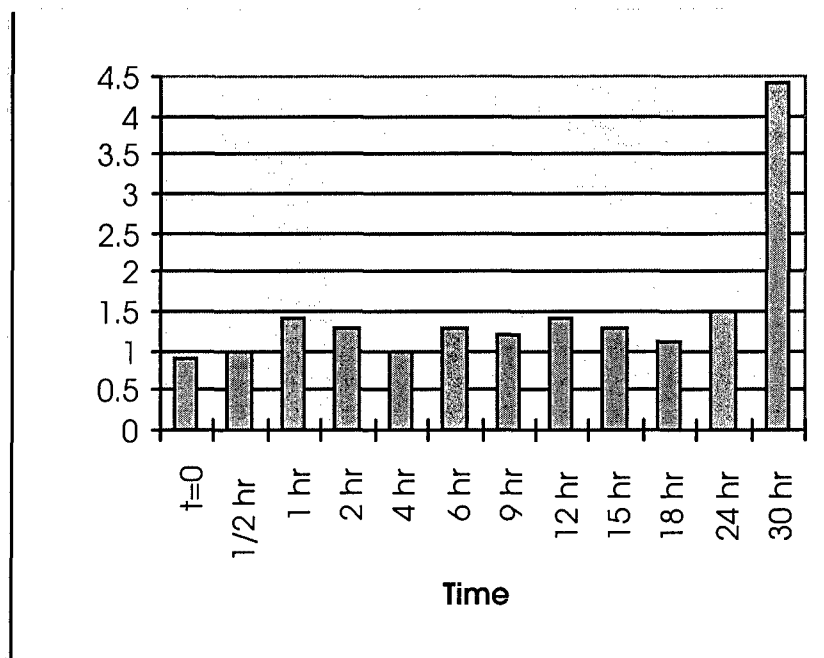


Figure 1. Krcr Immunoprecipitation (IP) and Western Analysis. IP's were performed with each of the four antisera raised against Krcr peptides (1000, 1001, 1002, 1003). All IP's performed used 2ug of affinity purified antisera and 500ug of cell lysate. Western analysis was performed with antisera 1000 and 1003 on the indicated samples. Both antisera recognize Krcr in cell lysates. 1000, 1002, and 1003 antisera can IP Krcr protein while the 1003 antisera appears to IP more Krcr protein than does 1000 or 1002.

**A.**



**B.**

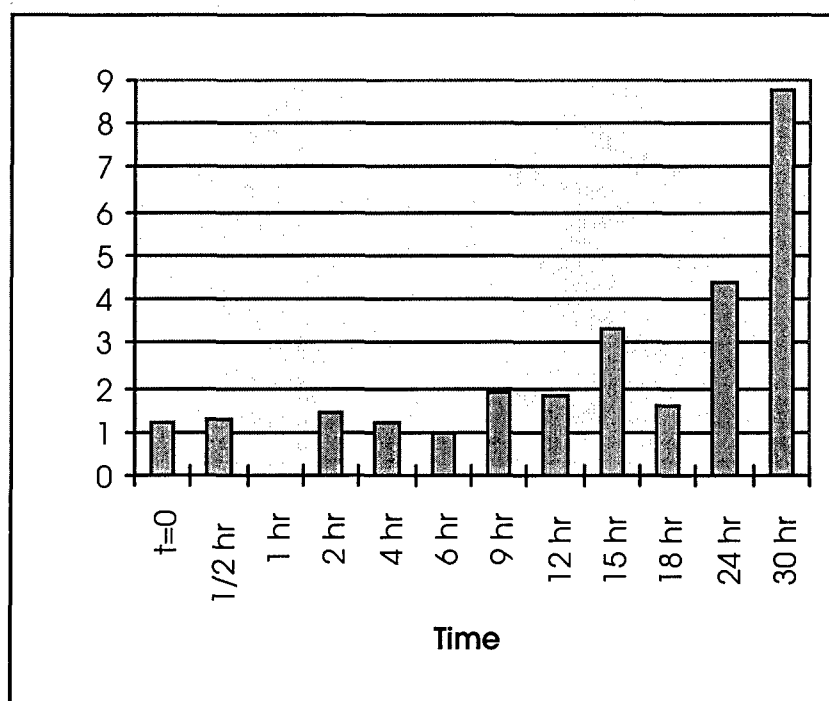


Figure 2. Krct Cell Cycle Analysis. Cells in both experiments were starved for 48 hours and then refed in media containing 10% serum. Cells were harvested at the various times indicated. Krct expression was normalized using ARPP-PO (A) Krct expression in HBI2 cells. Krct expression increases at 30 hours. (B) Krct expression in 16MB9A cells. Krct expression increases at 24 hours.

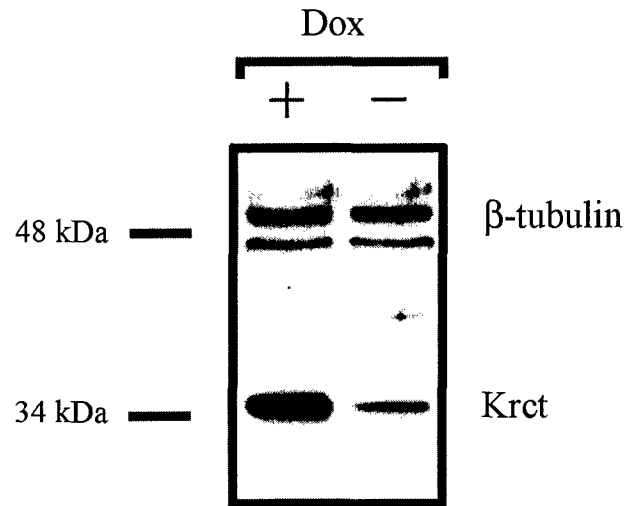


Figure 3. Doxycycline induction of a Krcr inducible clone. Western analysis was performed on a dox-inducible Krcr clone in the presence and absence of 1ug/ml of Doxycycline for 48 hours. 20 ug of cell extracts from induced and uninduced samples were analyzed with AB1003, a polyclonal antisera recognizing Krcr. Approximately 5 fold more protein is seen for Krcr in induced cell extracts when compared to uninduced extracts. Monoclonal antibodies recognizing β-tubulin were used to demonstrate equal loading.

### Key Research Accomplishments:

- Generated antisera to two independent regions of Krct which recognize Krct by Western analysis and Immunoprecipitation.
- Generated the TetO-Krct expression construct responsive to rtTA and tetracycline.
- Generated the MMTV-Krct expression construct for over-expression of Krct in the mammary gland of transgenic mice.
- Determined that Krct mRNA levels are regulated during the cell cycle in response to serum starvation/refeeding in two mammary epithelial cell lines.
- Generated three lines of mice carrying an MMTV-Krct transgene.
- Generated one HC-11 cell line clone in which Krct can be inducibly overexpressed in response to doxycycline.

### Reportable Outcomes:

- (1) Stairs DB, Perry Gardner H, Ha SI, Copeland NG, Gilbert DJ, Jenkins NA, Chodosh LA. Cloning and characterization of Krct, a member of a novel subfamily of serine/threonine kinases. *Hum Mol Genet* (1998) 7(13):2157-2166.
- (2) Chodosh LA, D'Cruz CM, Gardner HP, Ha SI, Marquis ST, Rajan JV, Stairs DB, Wang JY, Wang M. Mammary gland development, reproductive history, and breast cancer risk. *Cancer Res* (1999) 59(7 Suppl):1765-1771s; discussion 1771s-1772s.

### Conclusions:

Analysis of Krct expression using proliferation and differentiation assays as well as determining what affects over-expression of Krct has on these processes will yield valuable insight into Krct's potential role in mammary gland development and carcinogenesis. Preliminary results from analysis of Krct expression during the cell cycle demonstrate that Krct mRNA levels are regulated. Krct expression in the serum-starved state is low compared to actively dividing cells. Upon refeeding serum-starved cells, Krct expression rises dramatically just after Cyclin A induction in the cell cycle occurs. This finding, while suggestive of a possible role for Krct in proliferation, is not conclusive. Follow-up Western analysis experiments showed that Krct protein levels do not change during the cell cycle in HC-11 cells. Differences seen between Northern and Western analysis may be explained in several ways. Krct's 5'UTR contains elements known to regulate translational efficiency, and therefore protein levels need not reflect mRNA levels. Alternatively, different cell lines were used for protein and Northern analysis. While unlikely, Krct expression may differ during the cell cycle in these cell lines. Further experiments will address the disparate results seen. Use of the inducible Krct cell line will be valuable in determining if Krct might play a role in cell cycle regulation.

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